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L. Edelen

L. EDELEN
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INVENTOR(s)/APPLICANT(s)

GIVEN NAME (first and middle[if any])	FAMILY OR SURNAME	RESIDENCE (CITY AND EITHER STATE OR FOREIGN COUNTRY)
William C.	**Manning, Jr.**	**Redwood City, California**
Varavani J.	**Dwarki**	**Alameda, California**
Katherine	**Rendahl**	**Berkeley, California**
Shangzhen	**Zhou**	**Alameda, California**
Laura H.	**McGee**	**Oakland, California**
Dana	**Lau**	**Berkeley, California**
John G.	**Flannery**	**Berkeley, California**

jc541 U.S. PTO
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☐ Additional inventors are being named on separately numbered sheets attached hereto.

TITLE OF THE INVENTION (280 characters max)

USE OF RECOMBINANT GENE DELIVERY VECTORS FOR TREATING OR PREVENTING DISEASES OF THE EYE

CORRESPONDENCE ADDRESS

David D. McMasters
Seed and Berry LLP
6300 Columbia Center
701 Fifth Avenue
Seattle, Washington 98104-7092
Phone: (206) 622-4900 Fax: (206) 682-6031

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Respectfully submitted,

SIGNATURE David D. McMasters Date March 15, 1999

TYPED or PRINTED NAME DAVID D. MCMASTERS REGISTRATION NO. 33,963 *(if appropriate)*

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USE OF RECOMBINANT GENE DELIVERY VECTORS FOR TREATING OR PREVENTING DISEASES OF THE EYE

TECHNICAL FIELD

The present invention relates generally to compositions and methods for treating diseases of the eye, and more specifically, to the use of various gene delivery vectors which direct the expression of selected gene products suitable for treating or preventing retinal diseases of the eye.

BACKGROUND OF THE INVENTION

The retina, which is found at the back of the eye, is a specialized light-sensitive tissue that contains photoreceptor cells (rods and cones) and neurons connected to a neural network for the processing of visual information (see Figure 10). This information is sent to the brain for decoding into a visual image.

The retina depends on cells of the adjacent retinal pigment epithelium (RPE) for support of its metabolic functions. Photoreceptors in the retina, perhaps because of their huge energy requirements and highly differentiated state, are sensitive to a variety of genetic and environmental insults. The retina is thus susceptible to an array of diseases that result in visual loss or complete blindness.

Retinitis pigmentosa (RP), which results in the destruction of photoreceptor cells, the RPE, and choroid typifies inherited retinal degenerations. This group of debilitating conditions affects approximately 100,000 people in the United States.

A great deal of the progress made in addressing this important clinical problem has depended on advances in research on photoreceptor cell biology, molecular biology, molecular genetics, and biochemistry over the past two decades. Animal models of hereditary retinal disease have been vital in helping unravel the specific genetic and biochemical defects that underlie abnormalities in human retinal diseases. It now seems clear that both genetic and clinical heterogeneity underlie many hereditary retinal diseases.

The leading cause of visual loss in the elderly is Age-related Macular Degeneration (AMD). The social and economic impact of this disease in the United States is increasing. The macula is a structure near the center of the retina that contains the fovea. This specialized portion of the retina is responsible for the high-resolution vision that permits activities such as reading. The loss of central vision in AMD is devastating. Degenerative changes to the macula (maculopathy) can occur at almost

any time in life but are much more prevalent with advancing age. With growth in the aged population, AMD will become a more prevalent cause of blindness than both diabetic retinopathy and glaucoma combined. Laser treatment has been shown to reduce the risk of extensive macular scarring from the "wet" or neovascular form of the disease. The effects of this treatment are short-lived, however, due to recurrent choroidal neovascularization. Thus, there are presently no effective treatments in clinical use for the vast majority of AMD patients.

The present invention provides compositions and methods for treating and preventing a number of retinal diseases and degenerations such as RP and AMD, and further provides other related advantages.

SUMMARY OF THE INVENTION

Briefly stated, the present invention provides compositions and methods for treating, preventing, or, inhibiting diseases of the eye, and in particular, retinal diseases of the eye. Within one aspect of the present invention, methods are provided for treating or preventing diseases of the eye comprising the general step of intraocularly administering a gene delivery vector which directs the expression of one or more neurotrophic factors, such that the retinal disease of the eye is treated or prevented. Within related aspects of the present invention, gene delivery vectors are provided which direct the expression of a neurotrophic factor such as FGF. Within certain embodiments of the invention, a viral promoter (*e.g.*, CMV), a tissue-specific promoter (*e.g.*, opsin or RPE), or an inducible promoter (*e.g.*, tet) is utilized to drive the expresion of the neurotrophic factor.

Representative examples of gene delivery vectors suitable for use within the present invention may be generated from viruses such as retroviruses (*e.g.*, FIV or HIV), herpesviruses, adenoviruses, adeno-associated viruses, and alphaviruses, or from non-viral vectors.

Utilizing the methods and gene delivery vectors provided herein a wide variety of retinal diseases may be readily treated or prevented, including for example, macular degeneration, diabetic retinopathies, inherited retinal degeneration such as retinitis pigmentosa, glaucoma, retinal detachment or injury and retinopathies (whether inherited, induced by surgery, trauma, a toxic compound or agent, or, photically). Similarly, a wide variety of neurotrophic factors may be utilized (either alone or in combination) within the context of the present invention, including for example, NGF, BDNF, CNTF, NT-3, NT-4, FGF-2, FGF-5 and FGF-18.

Also provided by the present invention are isolated nucleic acid molecules comprising the sequence of Figure 2, vectors which contain, and/or express this sequence, and host cells which contain such vectors.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, various references are set forth herein which describe in more detail certain procedures or compositions (*e.g.*, plasmids, etc.), and are therefore incorporated by reference in their entirety.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic illustration of pKm201bFGF-2.

Figure 2 is the nucleic acid sequence of pKm201bFGF-2 (SEQ ID. No. ____).

Figure 3 is a schematic illustration of pD10-CMV-FGF-5.

Figure 4 is a western analysis of FGF-5 rAAV infected 293 cells.

Figure 5 is a schematic illustration of pD10-CMV-FGF-5 (sig-).

Figure 6 is a western analysis of pD10-CMV-FGF-5 (sig-) transfected 293 cells.

Figure 7 is a schematic illustration of pD10-CMV-kFGF-18.

Figure 8 western analysis of 293 cells transfected pD10-CMV-FGF-18.

Figures 9A and 9B are photographs which show that bluo-gal staining is visible across 40% of a retina transfected with AAV-CMV-lacZ. All photoreceptors appear to express lacZ at the injection site, except at the edge where individual cells are visible.

Figure 10 is a schematic illustration which shows the retina within the eye, and the organization of cells within the retina.

Figures 11A and 11B are photographs of wild-type and degenerated S334ter rat retinas. S334ter is a rat model for retinitis pigmentosa.

Figures 12A, 12B and 12C are photographs of degenerated S334ter, FGF-2 injected S334ter and PBS injected S334ter rat retinas. As can be seen in these figures, FGF-2 injected into the S334ter rat retina substantially slows the progression of disease.

Figure 13 is a graph which plots Outer Nuclear Layer (ONL) thickness forf FGF-2 subretinally injected, PBS subretinally injected, and an uninjected control.

Figure 14 is a bar graph which plots ONL thickness at p60.

Figures 15A, 15B and 15C are photographs of FGF-2 expressing cells stained with an anti-FGF-2 antibody.

DETAILED DESCRIPTION OF THE INVENTION

DEFINITIONS

Prior to setting forth the invention, it may be helpful to an understanding thereof to first set forth definitions of certain terms that will be used hereinafter.

"Gene delivery vehicle" refers to a construct which is capable of delivering, and, within preferred embodiments expressing, one or more gene(s) or sequence(s) of interest in a host cell. Representative examples of such vehicles include viral vectors, nucleic acid expression vectors, naked DNA, and certain eukaryotic cells (*e.g.*, producer cells).

"Recombinant adeno-associated virus vector"or "rAAV vector" refers to a gene delivery vector based upon an adeno-associated virus. The rAAV vectors, should contain 5' and 3' adeno-associated virus inverted terminal repeats (ITRs), and a transgene or gene of interest operatively linked to sequences which regulate its expression in a target cell. Within certain embodiments, the transgene may be operably linked to a hetereologous promoter (such as CMV), a tissue-specific promoter (*e.g.*, opsin or RPE), or an inducible promoter such as (tet). In addition, the rAAV vector may have a polyadenylation sequence.

"Neurotrophic Factor" or "NT" refers to proteins which are responsible for the development and maintenance of the nervous system. Representative examples of neurotrophic factors include NGF, BDNF, CNTF, NT-3, NT-4, and Fibroblast Growth Factors.

"Fibroblast Growth Factor" or "FGF" refers to a family of related proteins, the first of which was isolated from the pituitary gland (see Gospodarowicz, D., *Nature, 249*:123-127, 1974). From this original FGF (designated basic FGF) a family of related proteins, protein muteins, and protein analogs have been identified (see, *e.g.*, U.S. Patent Nos. 4,444,760, 5,155,214, 5,371,206, 5,464,774, 5,464,943, 5,604,293, 5,731,170, 5,750,365, 5,851,990, 5,852,177, 5,859,208, and 5,872,226), all of which are generally referred to as Fibroblast Growth Factors within the context of the present invention.

As noted above, the present invention provides compositions and methods for treating, preventing, or, inhibiting retinal diseases of the eye, comprising the general step of administering intraocularly a recombinant adeno-associated viral vector which directs the expression of one or more neurotrophic factors, such that the

retinal disease of the eye is treated or prevented. In order to further an understanding of the invention, a more detailed discussion is provided below regarding (A) gene delivery vectors; (B) Neurotrohpic Factors; and (C) methods of administering the rAAVs in the treatment or prevention of retinal diseases of the eye.

A. Gene Delivery Vectors

1. Construction of retroviral gene delivery vectors

Within one aspect of the present invention, retroviral gene delivery vehicles are provided which are constructed to carry or express a selected gene(s) or sequence(s) of interest. Briefly, retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses, including for example, B, C, and D type retroviruses as well as spumaviruses and lentiviruses (see RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS 82*:488, 1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, within one embodiment of the invention, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

Within one aspect of the present invention, retrovector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks *gag/pol* or *env* coding sequences.

Other retroviral gene delivery vehicles may likewise be utilized within the context of the present invention, including for example EP 0,415,731; WO 90/07936; WO 91/0285, WO 9403622; WO 9325698; WO 9325234; U.S. Patent No. 5,219,740; WO 9311230; WO 9310218; Vile and Hart, *Cancer Res. 53*:3860-3864, 1993; Vile and Hart, *Cancer Res. 53*:962-967, 1993; Ram et al., *Cancer Res. 53*:83-88,

1993; Takamiya et al., *J. Neurosci. Res. 33*:493-503, 1992; Baba et al., *J. Neurosurg. 79*:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242 and WO91/02805).

Packaging cell lines suitable for use with the above described retrovector constructs may be readily prepared (see U.S. Serial No. 08/240,030, filed May 9, 1994; see also U.S. Serial No. 07/800,921, filed November 27, 1991), and utilized to create producer cell lines (also termed vector cell lines or "VCLs") for the production of recombinant vector particles.

2. Recombinant Adeno-Associated Virus Vectors

As noted above, a variety of rAAV vectors may be utilized to direct the expression of one or more desired neurotrophic factors. Briefly, the rAAV should be comprised of, in order, a 5' adeno-associated virus inverted terminal repeat, a transgene or gene of interest operatively linked to a sequence which regulates its expression in a target cell, and a 3' adeno-associated virus inverted terminal repeat. In addition, the rAAV vector may preferably have a polyadenylation sequence.

Generally, rAAV vectors should have one copy of the AAV ITR at each end of the transgene or gene of interest, in order to allow replication, packaging, and efficient integration into cell chromosomes. The ITR consists of nucleotides 1 to 145 at the 5' end of the AAV DNA genome, and nucleotides 4681 to 4536 (i.e., the same sequence) at the 3' end of the AAV DNA genome. Preferably, the rAVV vector may also include at least 10 nucleotides following the end of the ITR (i.e., a portion of the "D region").

Within preferred embodiments of the invention, the transgene sequence will be of about 2 to 5 kb in length (or alternatively, the transgene may additionally contain a "stuffer" or "filler" sequence to bring the total size of the nucleic acid sequence between the two ITRs to between 2 and 5 kb). Alternatively, the transgene may be composed of same heterologous sequence several times (*e.g.*, two nucleic acid molecules which encode FGF-2 separated by a ribosome readthrough, or alternatively, by an Internal Ribosome Entry Site or "IRES"), or several different heterologous sequences (*e.g.*, FGF-2 and FGF-5, separated by a ribosome readthrough or an IRES). In the latter instance where heterologous sequences are used, they may be organized as two separate transcription units each with its own promoter and polyadenylation signal.

Recombinant AVV vectors of the present invention may be generated from a variety of adeno-associated viruses, including for example, serotypes 1 through 6. For example, ITRs from any AAV serotype are expected to have similar structures

and functions with regard to replication, integration, excision and transcriptional mechanisms.

Within certain embodiments of the invention, expression of the transgene may be accomplished by a separate promoter (*e.g.*, a viral promoter). Representative examples of suitable promoters in this regard include a CMV promoter, RSV promoter, SV40 promoter, or MoMLV promoter. Other promoters that may similarly be utilized within the context of the present invention include cell or tissue specific promoters (*e.g.*, a rod, cone, or ganglia derived promoter), or inducible promoters. Representative examples of suitable inducible promoters include tetracycline-response promoters ("Tet", see, *e.g.*, Gossen and Bujard, *Proc. Natl. Acad. Sci. USA. 89*:5547-5551, 1992; Gossen et al., *Science 268*, 1766-1769, 1995; Baron et al., *Nucl. Acids Res. 25*:2723-2729, 1997; Blau and Rossi, *Proc. Natl. Acad. Sci. USA. 96*:797-799, 1999; Bohl et al., *Blood 92*:1512-1517, 1998; and Haberman et al., *Gene Therapy 5*:1604-1611, 1998), the ecdysone system (see *e.g.*, No et al., *Proc. Natl. Acad. Sci. USA. 93*:3346-3351, 1996), and other regulated promoters or promoter systems (see, *e.g.*, Rivera et al., *Nat. Med. 2*:1028-1032, 1996;).

The rAVV vector may also contain additional sequences, for example from an adenovirus, which assist in effecting a desired function for the vector. Such sequences include, for example, those which assist in packaging the rAVV vector in adenovirus particles.

Packaging cell lines suitable for producing adeno-associated viral vectors may be readily accomplished given readily available techniques (see *e.g.*, U.S. Patent No. 5,872,005).

Particularly preferred methods for constructing and packaging rAVV vectors are described in more detail below in Examples 1, 2, 3, and 4.

3. Alphavirus delivery vehicles

The present invention also provides a variety of Alphavirus vectors which may function as gene delivery vehicles. For example, the Sindbis virus is the prototype member of the alphavirus genus of the togavirus family. The unsegmented genomic RNA (49S RNA) of Sindbis virus is approximately 11,703 nucleotides in length, contains a 5' cap and a 3' poly-adenylated tail, and displays positive polarity. Infectious enveloped Sindbis virus is produced by assembly of the viral nucleocapsid proteins onto the viral genomic RNA in the cytoplasm and budding through the cell membrane embedded with viral encoded glycoproteins. Entry of virus into cells is by endocytosis through clatharin coated pits, fusion of the viral membrane with the

endosome, release of the nucleocapsid, and uncoating of the viral genome. During viral replication the genomic 49S RNA serves as template for synthesis of the complementary negative strand. This negative strand in turn serves as template for genomic RNA and an internally initiated 26S subgenomic RNA. The Sindbis viral nonstructural proteins are translated from the genomic RNA while structural proteins are translated from the subgenomic 26S RNA. All viral genes are expressed as a polyprotein and processed into individual proteins by post translational proteolytic cleavage. The packaging sequence resides within the nonstructural coding region, therefore only the genomic 49S RNA is packaged into virions.

Several different Sindbis vector systems may be constructed and utilized within the present invention. Representative examples of such systems include those described within U.S. Patent Nos. 5,091,309 and 5,217,879, and PCT Publication No. WO 95/07994.

4. Other viral gene delivery vectors

In addition to retroviral vectors and alphavirus vectors, numerous other viral vectors systems may also be utilized as a gene delivery vehicle. Representative examples of such gene delivery vehicles include viruses such as pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330 and 5,017,487; WO 89/01973); SV40 (Mulligan et al., *Nature 277*:108-114, 1979); influenza virus (Luytjes et al., *Cell 59*:1107-1113, 1989; McMicheal et al., *N. Eng. J. Med. 309*:13-17, 1983; and Yap et al., *Nature 273*:238-239, 1978); herpes (Kit, *Adv. Exp. Med. Biol. 215*:219-236, 1989; U.S. Patent No. 5,288,641); HIV (Poznansky, *J. Virol. 65*:532-536, 1991); measles (EP 0 440,219); Semliki Forest Virus, and coronavirus, as well as other viral systems (*e.g.*, EP 0,440,219; WO 92/06693; U.S. Patent No. 5,166,057). In addition, viral carriers may be homologous, non-pathogenic(defective), replication competent virus (*e.g.*, Overbaugh et al., *Science 239*:906-910,1988), and nevertheless induce cellular immune responses, including CTL.

5. Non-viral gene delivery vehicles

In addition to the above viral-based vectors, numerous non-viral gene delivery vehicles may likewise be utilized within the context of the present invention. Representative examples of such gene delivery vehicles include direct delivery of nucleic acid expression vectors, naked DNA alone (WO 90/11092), polycation condensed DNA linked or unlinked to killed adenovirus (Curiel et al., *Hum. Gene Ther.*

3:147-154, 1992), DNA ligand linked to a ligand with or without one of the high affinity pairs described above (Wu et al., *J. of Biol. Chem 264*:16985-16987, 1989), nucleic acid containing liposomes (*e.g.*, WO 95/24929 and WO 95/12387) and certain eukaryotic cells (*e.g.*, producer cells - see U.S. Serial No. 08/240,030, filed May 9, 1994, and U.S. Serial No. 07/800,921).

B. Neurotrophic Factors

As noted above, the term neurotrophic factor refers to proteins which are responsible for the development and maintenance of the nervous system. Representative examples of neurotrophic factors include NGF, BDNF, CNTF, NT-3, NT-4, and Fibroblast Growth Factors.

Fibroblast Growth Factor refers to a family of related proteins, the first of which was isolated from the pituitary gland (see Gospodarowicz, D., *Nature, 249*:123-127, 1974). From this original FGF (designated basic FGF) a family of related proteins, protein muteins, and protein analogs have been identified (see, *e.g.*, U.S. Patent Nos. 4,444,760, 5,155,214, 5,371,206, 5,464,774, 5,464,943, 5,604,293, 5,731,170, 5,750,365, 5,851,990, 5,852,177, 5,859,208, and 5,872,226; see generally Baird and Gospodarowicz, *D. Ann N.Y. Acad. Sci. 638*:1, 1991. The first two members of the family to be identified were acidic fibroblast growth factor (aFGF/FGF-1) and basic fibroblast growth factor (bFGF/FGF-2). Additional members of the FGF family include: i-nt-2/FGF-3, (Smith et al., *EMBO J.* 7: 1013, 1988); FGF-4 (Delli-Bovi et al., Cell 50: 729, 1987); FGF-6 (Marics et al., *Oncogene 4*: 335 (1989); keratinocyte growth factor/FGF-7, (Finch et al., *Science 245*: 752, 1989); FGF-8 (Tanaka et al., *Proc. Natl. Acad Sci. USA 89*: 8928, 1992); and FGF-9 (Miyamoto et al., *Mol. Cell Biol. 13*: 4251, 1993).

FGF-5 was originally isolated as an oncogene. See Goldfarb et al. US Patent Nos. 5,155,217 and 5,238,916, Zhan et al. "Human Oncogene Detected by a Defined Medium Culture Assay" (Oncogene (1987) 1:369-376), Zhan et al. "The Human FGF-5 Oncogene Encodes a Novel Protein Related to Fibroblastic Growth Factors" (Molecular and Cellular Biology (1988) 8:3487-3495), and Bates et al. "Biosynthesis of Human Fibroblast Growth Factor 5": (Molecular and Cellular Biology, (1991) 11:1840-1845).

Other FGFs include those disclosed in U.S. Patent Nos. 4,444,760, 5,155,214, 5,371,206, 5,464,774, 5,464,943, 5,604,293, 5,731,170, 5,750,365, 5,851,990, 5,852,177, 5,859,208, and 5,872,226.

C. Method for Treating and/or Preventing Retinal Disease, and Pharmaceutical Compositions

As noted above, the present invention provides methods which generally comprise the step of intraocularly administering a gene delivery vector which directs the expression of one or more neurotrophic factor to the retina in order to treat, prevent, or inhibit the progression of a retinal disease. As utilized herein, it should be understood that the terms "treated, prevented, or, inhibited" refers to the alteration of a disease course or progress in a statisically significant manner. Determination of whether a disease course has been altered may be readily assessed in a variety of model systems, discussed in more detail below, which analyze the ability of a gene delivery vector to delay, prevent or rescue photoreceptors, as well as other retinal cells, from cell death.

1. Retinal Diseases of the Eye

A wide variety of retinal diseases may be treated given the teachings provided herein. For example, within one embodiment of the invention gene delivery vectors are administered to a patient intraocularly in order to treat or prevent macular degeneration. Briefly, the leading cause of visual loss in the elderly is macular degeneration (MD), which has an increasingly important social and economic impact in the United States. As the size of the elderly population increases in this country, age related macular degeneration (AMD) will become a more prevalent cause of blindness than both diabetic retinopathy and glaucoma combined. Although laser treatment has been shown to reduce the risk of extensive macular scarring from the "wet" or neovascular form of the disease, there are currently no effective treatments for the vast majority of patients with MD.

Within other embodiments, gene delivery vectors can be administered to a patient intraocularly in order to treat or prevent diabetic retinopathy, or other vascular diseases of the retina.

Within another embodiment, gene delivery vectors can be administered to a patient intraocularly in order to treat or prevent an inherited retinal degeneration. One of the most common inherited retinal degenerations is retinitis pigmentosa (RP), which results in the destruction of photoreceptor cells, and the RPE. Other inherited conditions include Bardet-Biedl syndrome (autosomal recessive); Congenital amaurosis (autosomal recessive); Cone or cone-rod dystrophy (autosomal dominant and X-linked forms); Congenital stationary night blindness (autosomal dominant, autosomal recessive and X-linked forms); Macular degeneration (autosomal dominant and autosomal recessive forms); Optic atrophy, autosomal dominant and X-linked forms); Retinitis pigmentosa (autosomal dominant, autosomal recessive and X-linked forms); Syndromic

or systemic retinopathy (autosomal dominant, autosomal recessive and X-linked forms); and Usher syndrome (autosomal recessive). This group of debilitating conditions affects approximately 100,000 people in the United States alone.

Within other embodiments of the invention, gene delivery vectors can be administered to a patient intraocularly in order to treat or prevent glaucoma. Briefly, glaucoma is not a uniform disease but rather a heterogeneous group of disorders that share a distinct type of optic nerve damage that leads to loss of visual function. The disease is manifest as a progressive optic neuropathy that, if left untreated leads to blindness. It is estimated that as many as 3 million Americans have glaucoma and, of these, as many as 120,000 are blind as a result. Furthermore, it is the number one cause of blindness in African-Americans. Its most prevalent form, primary open-angle glaucoma, can be insidious. This form usually begins in midlife and progresses slowly but relentlessly. If detected early, disease progression can frequently be arrested or slowed with medical and surgical treatment.

Within yet other embodiments gene delivery vectors can be administered to a patient intraocularly in order to treat or prevent injuries to the retina, including retinal detachment, photic retinopathies, surgery-induced retinopathies, toxic retinopathies, retinopathies due to trauma or penetrating lesions of the eye.

2. Methods of Administration

Gene delivery vectors of the present invention may be administered intraocularly to a variety of locations. Briefly, the human retina is organized in a fairly exact mosaic. In the fovea, the mosaic is a hexagonal packing of cones. Outside the fovea, the rods break up the close hexagonal packing of the cones but still allow an organized architecture with cones rather evenly spaced surrounded by rings of rods. Thus in terms of densities of the different photoreceptor populations in the human retina, it is clear that the cone density is highest in the foveal pit and falls rapidly outside the fovea to a fairly even density into the peripheral retina (see Osterberg, G. (1935) Topography of the layer of rods and cones in the human retina. Acta Ophthal.(suppl.) 6, 1-103; see also Curcio, C. A., Sloan, K. R., Packer, O., Hendrickson, A. E. and Kalina, R. E. (1987) Distribution of cones in human and monkey retina: individual variability and radial asymmetry. Science 236, 579-582).

Access to desired portions of the retina, or to other parts of the eye may be readily accomplished by one of skill in the art (see generally Medical and surgical retina : advances, controversies, and management, editors, Hilel Lewis, Stephen J. Ryan; medical illustrator, Timothy C. Hengst. St. Louis : Mosby, c1994. xix, 534 p. :

ill. ; 28 cm; see also Retina , editor in chief, Stephen J. Ryan. 2nd ed. St. Louis : Mosby, c1994. 3 v. (xxix, 2559 p.) : ill. ; 29 cm.).

The amount of the specific viral vector applied to the retina is uniformly quite small as the eye is a relatively contained structure and the agent is injected directly into it. The amount of vector that needs to be injected is determined by the intraocular location of the chosen cells targeted for treatment. The cell type to be transduced will be determined by the particular disease entity that is to be treated.

For example, a single 20-microliter volume (of 10^{13} per milliliter physical particle titer rAAV) may be used in a subretinal injection to treat the macula and fovea. A larger injection of 50 to 100 microliters may be used to deliver the rAAV to a substantial fraction of the retinal area, perhaps to the entire retina depending upon the extent of lateral spread of the particles.

A 100-ul injection will provide several million active rAAV particles in to the subretinal space. This calculation is based upon a titer of 10^{13} physical particles per milliliter. Of this titer, it is estimated that 1/1000 to 1/10,000 of the AAV particles are infectious. The retinal anatomy constrains the injection volume possible in the subretinal space (SRS). Assuming an injection maximum of 100 microliters, this would provide an infectious titer of 10^{8} to 10^{9} rAAV in the SRS. This would have the potential of infecting all of the ~150 x 10^{6} photoreceptors in the entire human retina with a single injection.

Smaller injection volumes focally applied to the fovea or macula may adequately transfect the entire region affected by the disease in the case of macular degeneration or other regional retinopathies.

Gene delivery vectors can alternately be delivered to the eye by intraocular injection into the vitreous. In this application, the primary target cells to be transduced are the retinal ganglion cells, which are the retinal cells primarily affected in glaucoma. In this application, the injection volume of the gene delivery vector could be substantially larger, as the volume is not constrained by the anatomy of the interphotoreceptor or subretinal space. Acceptable dosages in this instance can range from 25 ul to 1000ul.

3. Assays

A wide variety of assays may be utilized in order to determine appropriate dosages for administration, or to assess the ability of a gene delivery vector to treat or prevent a particular disease. Certain of these assays are discussed in more detail below.

a. Electroretinographic analysis

Electroretinographic analysis can be utilized to assess the effect of gene delivery administration into the retina. Briefly, rats are dark adapted overnight and then in dim red light, then anesthetized with intramuscular injections of xylazine (13 mg/kg) and ketamine (87 mg/kg). Full-field scotopic ERGs are elicited with 10-μsec flashes of white light and responses were recorded using a UTAS-E 2000 Visual Electrodiagnostic System (LKC Technologies, Inc., Gaithersburg, MD). The corneas of the rats are the anesthetized with a drop of 0.5% proparacaine hydrochloride, and the pupils dilated with 1% atropine and 2.5% phenylephrine hydrochloride. Small contact lenses with gold wire loops are placed on both corneas with a drop of 2.5% methylcellulose to maintain corneal hydration. A silver wire reference electrode is placed subcutaneously between the eyes and a ground electrode is placed subcutaneously in the hind leg. Stimuli are presented at intensities of -1.1, 0.9 and 1.9 log cd m^{-2} at 10-second, 30-second and 1-minute intervals, respectively. Responses are amplified at a gain of 4,000, filtered between 0.3 to 500 Hz and digitized at a rate of 2,000 Hz on 2 channels. Three responses are averaged at each intensity. The a-waves are measured from the baseline to the peak in the cornea-negative direction, and b-waves are measured from the cornea-negative peak to the major cornea-positive peak. For quantitative comparison of differences between the two eyes of rats, the values from all the stimulus intensities are averaged for a given animal.

b. Retinal tissue analysis

Retinal tissue analysis can also be utilized to assess the effect of gene delivery administration into the retina. This procedure is described in more detail below in Example 7.

4. Pharmaceutical Compositions

Gene delivery vectors may be prepared as a pharmaceutical product suitable for direct administration. Within preferred embodiments, the vector should be admixed with a pharmaceutically acceptable carrier for intraocular administration. Examples of suitable carriers are saline or phosphate buffered saline.

DEPOSIT INFORMATION

The following material was deposited with he American Type Culture Collection:

Name	Deposit Date	Accession No.
PKm201bFGF-2		

The above material was deposited by Chiron Corporation with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, VA 20110-2209, telephone 703-365-2700. This deposit will be maintained under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for purposes of Patent Procedure. The deposit will be maintained for a period of 30 years following issuance of this patent, or for the enforceable life of the patent, whichever is greater. Upon issuance of the patent, the deposits will be available to the public from the ATCC without restriction.

This deposit is provided merely as a convenience to those of skill in the art, and is not an admission that a deposit is required under 35 U.S.C. § 112. The nucleic acid sequence of this deposit, as well as the amino acid sequence of the polypeptide encoded thereby, are incorporated herein by reference and should be referred to in the event of an error in the sequence described herein. A license may be required to make, use, or sell the deposited materials, and no such license is granted hereby.

The following examples are offered by way of illustration, and not by way of limitation.

EXAMPLES

EXAMPLE 1

CONSTRUCTION OF A rAAV VECTOR EXPRESSING FGF-2

pKm201CMV is an AAV cloning vector in which an expression cassette, consisting of a CMV immediate early promoter/enhancer and a bovine growth hormone (BGH) polyadenylation site, is flanked by inverted terminal repeat (ITR) sequences from AAV-2. Briefly, pKm201CMV was derived from pKm201, a modified AAV vector plasmid in which the ampicillin resistance gene of pEMBL-AAV-ITR (see Srivastava, (1989) Proc. Natl. Acad. Sci. USA 86:8078-8082) had been replaced with the gene for kanamycin resistance. The expression cassette from pCMVlink, a derivative of pCMV6c (see Chapman, (1991) Nucleic Acids Res. 19:193-198) in which the BGH poly A site has been substituted for the SV40 terminator, was inserted between the ITRs of pKm201 to generate pKm201CMV.

pKm201bFGF-2 was constructed by cloning the following, in order, into the multiple cloning site of pKm201CMV: the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES), the bovine FGF-2 cDNA, and the human growth hormone polyadenylation sequence. The cDNA for FGF-2 has two mutations that change amino acid 121 from serine to threonine and amino acid 137 from proline to serine. The DNA sequence of pKm201bFGF-2 is shown in Figure 2 and the plasmid has been deposited with the American Type Culture Collection (ATCC).

rAAV vector particles were produced by a triple transfection protocol (*Nucleic Acids Res. 24*:596-601, 1996; *J. Exp. Med. 179*:1867-1875, 1994). Briefly, human embryonic kidney 293 cells, grown to 50% confluence in a 10 layer Nunclon cell factory (Nalge Nunc, Int., Naperville, IL), were co-transfected with 400 μg of helper plasmid pKSrep/cap (*Hum. Gene Ther. 9*:477-485, 1998) 400 μg of vector plasmid, and 800 μg of adenovirus plasmid pBHG10 (Microbix Biosystems, Inc., Toronto, Ontario) using the calcium phosphate co-precipitation method. Forty-eight hours after co-transfection, media was replaced with IMDM + 10% FBS containing adenovirus type 5 dl312 at a multiplicity of infection (MOI) of 2. Seventy-two hours after infection cells were harvested and resuspended in HEPES buffer (2.5 ml per dish) and lysed by three cycles of freezing and thawing. Cell debris was removed by centrifugation at 12,000X g for 20 min. Packaged rAAV was purified from adenovirus by two rounds of cesium chloride equilibrium density gradient centrifugation. Residual adenovirus contamination was inactivated by heating at 56°C for 45 min.

To estimate total number of rAAV particles, the virus stock was treated with DNAse I, and encapsidated DNA was extracted with phenol-chloroform, and precipitated with ethanol. DNA dot blot analysis against a known standard was used to determine titer (*Blood 76*:1997-2000, 1990).

To assay for adenovirus contamination, 293 cells were infected with 10 µl of purified rAAV stock and followed for any signs of cytopathic effect. All stocks were negative for adenovirus contamination (level of detection greater than or equal to 100 PFU/ml).

To assay for wild type AAV, 293 cells were co-infected with serial dilutions of rAAV stocks and adenovirus dl312 at a MOI of 2. Three days later the cells were harvested, lysed by three cycles of freezing/thawing, and centrifuged to remove cell debris. The supernatant was heat inactivated (56°C for 10 min) and fresh plates of 293 cells (6×10^6) were infected in the presence of adenovirus dl312 at a MOI of 2. Forty-eight hours after infection, low molecular-weight DNA was isolated (*J. Mol. Biol. 26*:365-369, 1967) subjected to agarose gel electrophoresis, and transferred to a nylon membrane. The membrane was hybridized with a biotinylated oligonucleotide probe specific for the AAV capsid region. The wild type AAV titer was defined as the highest dilution of rAAV vector stock demonstrating a positive hybridization signal. The rAAV preparations contained less than 1 wild type AAV genome per 10^9 rAAV genomes.

EXAMPLE 2

INFECTION OF CELLS WITH RAAV-CMV-FGF-2 RESULTS IN THE EXPRESSION OF FGF-2

293 cells were plated the day before infection at 5×10^5 cells/well in 6-well plates and were infected with rAAV-CMV-bFGF-2 virus, prepared as described above in Example 1, at different multiplicities of infection (MOI) with and without etoposide (3µM). Etoposide is a topoisomerase inhibitor which has been shown to increase transduction efficiency of rAAV vectors (*Proc. Natl. Acad. Sci. USA, 92*:5719-5723, 1995). Forty-eight hours after infection, culture supernatant was collected and cells were lysed in 0.5 mL 1x lysis buffer (100 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA, 0.5% NP40, and 0.5% deoxycholate). FGF-2 in the supernatants and lysates was assayed by ELISA (cat. # DFB00, R & D Systems, Minneapolis, MN) following manufacturer's instructions. The results are shown below in Table 1.

TABLE 1

FGF-2 production in 293 cells following infection with rAAV-FGF-2

Culture medium supernatant (1.5 ml)

sample	infection MOI	Etoposide	FGF2 protein
1	0	-	<5 pg/ml
2	$2X10^5$	-	<5 pg/ml
3	$2X10^4$	-	<5 pg/ml
4	$2X10^3$	-	<5 pg/ml
1	0	+	<5 pg/ml
2	$2X10^5$	+	106 pg/ml ≈ 300pg/24h/10^6cells
3	$2X10^4$	+	<5 pg/ml
4	$2X10^3$	+	<5 pg/ml

Cell lysate (0.5 ml)

sample	infection MOI	Etoposide	FGF2 protein
1	0	-	8.95 ng/ml
2	$2X10^5$	-	114. ng/ml
3	$2X10^4$	-	18.8 ng/ml
4	$2X10^3$	-	11.3 ng/ml
1	0	+	5.05 ng/ml
2	$2X10^5$	+	296. ng/ml ≈ 300ng/24h/10^6cells
3	$2X10^4$	+	48.0 ng/ml
4	$2X10^3$	+	13.2 ng/ml

EXAMPLE 3

CONSTRUCTION OF pD10-bFGF-2

The D10 AAV vector is constructed by replacing the AAV gene encoding sequences of pD-10, (see Wang, X. et al. *J. Virol.* 71:3077 (1997), with the CMV promoter, multiple cloning site, and BGH polyadenylation sequences from pKm201CMV. Briefly, oligonucleotides 5'-ggtatttaaa acttgcggcc gcggaatttc gactctaggc c-3' (SEQ I.D. No. ____) and 5'-gctgcccggg acttgctagc tggatgatcc tccagcgcgg ggatctcatg -3' (SEQ I.D. No. ____) are used to amplify the CMV expression cassette from pKm201CMV. The product of this PCR amplification is digested with SmaI and DraI and cloned into pD-10 digested with EcoRV. This new vector is named pD10-CMV.

To construct pD10-bFGF-2, the synthetic gene for bovine FGF-2 (see US Patent 5,464,774 for sequence) is digested with EcoRI and SalI, treated with T4 polymerase to blunt the ends, and then cloned into the StuI site of pD10-CMV. The synthetic gene described above encodes the mature, processed form of bovine FGF-2.

EXAMPLE 4

CONSTRUCTION OF rAAV VECTORS WHICH EXPRESS FGF5 AND FGF18

A. Cloning FGF-5 into the pD10-CMV rAAV Vector.

The FGF-5 coding region (see U.S. Serial No. 08,602,147) was cloned into the rAAV pD10-CMV vector by digestion with the enzymes SacII and XmnI, resulting in an 814 bp fragment. This removed an ORF (ORF-1) upstream of and overlapping with the FGF-5 coding region. The ends of the FGF-5 fragment were then blunted with T4 DNA polymerase, and it was cloned into the rAAV pD10-CMV vector linearized with StuI. This vector contains a 1353 bp insertion of a bacteriophage Phi X174 HaeIII fragment.

The pD10-CMV-FGF-5 vector is illustrated schematically in Figure 3. In summary, this plasmid contains the CMV immediate/early enhancer + promoter, the CMV intron A, an FGF-5 coding region, the bovine growth hormone polyA site, and AAV ITR sequences. There is a 1353 bp insertion of PhiX 174 bacteriophage DNA clones into the NotI site between one ITR and the CMV immediate early enhancer + promoter region.

B. Packaging and Analysis of FGF-5 rAAV Expression.

rAAV virus was packaged using a triple transfection method. Briefly, 8 x 10^8 or 8 x 10^9 particles of the resulting FGF-5 virus were used to infect 293 cells, which were simultaneously treated with 3 uM etoposide to enhance viral expression levels. At 24 hours post-infection, tissue culture media and cell lysates were harvested and analysed by Western blotting. Briefly, protein samples were run on a 4-20% tris-glycine gradient gel, and transferred to nitrocellulose by standard procedures. After blocking with 5% milk in PBS, the membrane was incubated with an anti-human FGF-5 antibody (R and D systems, made in goat) at a dilution of 1,000 for one hour at room temperature. After the membrane was washed 3 times in PBS + 0.05% Tween-20, it was incubated with an anti-goat secondary antibody conjugated to peroxidase (1:5,000 dilution). The membrane was then washed and the FGF-5 protein detected by chemiluminescence.

Results of the Western blot are shown in Figure 4. Briefly, lane 1 represents 50 ng of the 29.5 Kd recombinant FGF-5 protein (R and D systems). Lane 2, media from cells infected with 8 x 10^9 viral particles and treated with etoposide, shows no detectable FGF-5 expression. Lane 3 is an uninfected cell lysate control. Lane 4 and 5 are lysates from cells infected with 8 x 10^8 or 8 x 10^9 viral particles, respectively, and Lanes 6 and 7 are lysates from cells infected with 8 x 10^8 or 8 x 10^9 viral particles and treated with 3 uM etoposide. Lanes 4-7 all show positive FGF-5 expression. Lane 8 is a negative control of lysate from unifected cells.

In summary, although the FGF-5 signal sequence was intact, FGF-5 protein was detected in the cell lysate only.

C. Cloning FGF-5 Lacking a Signal Sequence, into rAAV pD10-CMV.

Mitogenic activity is associated with the wild-type FGF-5 molecule (Zhan et al., 1988; Bates et al., 1991). To improve its safety, the codons for the first 21 amino acids of FGF-5's signal sequence were removed by PCR amplification of the above pD10-CMV-FGF-5 plasmid with the following primers: AGA/TAT/AAG/CTT/AC C/ATG/GGT/GAA/AAG/CG T/CTC/GCC/CCC/AAA (5', 5FGFMUTB; SEQ I.D. No._) and CGC/GCG/CTC/GAG/AC C/ATG/AGG/AAT/ATT/AT C/CAA/AGC/GAA/ACT (3', 3FGF5WT; SEQ I.D. No. ____). The 5' primer contains two silent mutations which should destabilize G/C rich hairpin structures of the FGF-5 mRNA, and should increase the efficiency of translation and thus, gene expression. The PCR product was digested with HindIII and XhoI (sites introduced by the primers), and cloned by standard methods, into the D10 vector

digested with the same enzymes. The pD10-CMV-FGF-5 (sig-) vector is illustrated schematically in Figure 5.

In summary, the pD10-CMV-FGF-5 (sig-) plasmid contains the CMV immediate/early enhancer + promoter, the CMV intron A, the FGF-5 coding region with the modifications described in Example C above, the bovine growth hormone polyA site, and the AAV ITR sequences. There is a 1353 bp insertion of PhiX 174 bacteriophage DNA cloned into the NotI site between one ITR and the CMV immediate early enhancer + promoter region.

D. Western Analysis of 293 Cells Transfected with pD10-CMV-FGF-5 (sig-).

Expression of FGF-5 protein was demonstrated by transient transfection of 293 cells with the plasmid pD10-CMV-FGF-5 (sig-), by standard methods. After 48 hours, tissue culture media and cell lysates were harvested. Western analysis was performed with an anti-human FGF-5 antibody (R and D systems) as described above.

Results of the western analysis are provided below in Figure 6. Briefly, lane 1 represents 50 ng of the 29.5 Kd recombinant FGF-5 protein (R and D systems). Lanes 2 and 3, showing FGF-5 expression, are cell lysates from 293 cells transfected with two different clones of the pD10-CMV-FGF-5 sig- plasmid. Lane 4 is lysate from cells transfected with a negative control plasmid CMV-Epo. Lanes 5, 6 and 7 represent media from cells transfected with different clones of the pD10-CMV-FGF-5 sig- plasmid, respectively, and the CMV-Epo plasmid. As is evident from this figure, FGF-5 protein was detected in the cell lysate only.

E. Generation of FGF-5 (signal -) rAAV.

FGF-5 (sig-) rAAV virus is packaged using the triple transfection method described in more detail above.

F. Cloning FGF-18 into the pD10-CMV rAAV Vector.

The FGF-18 coding region (see U.S. Provisional Application Serial No. 60/083,553) was cloned into the pD10-CMV vector as a PstI to EcoRV fragment, using restriction sites found in both the FGF-18 and the multiple cloning site of the pD10-CMV vector. The vector contains a 1353 bp insertion of PhiX174 bacteriophage DNA (see Example A).

A schematic illustration of pD10-CMV-FGF-18 is provided in Figure 7. Briefly, this plasmid contains the CMV immediate/early enhancer + promoter, the CMV intron A, the FGF-18 coding region, the bovine growth hormone polyA site, and the

AAV ITR sequences. There is a 1353 bp insertion of PhiX 174 bacteriophage DNA cloned into the Not1 site between one ITR and the CMV immediate early enhancer + promoter region.

G. Analysis of 293 Cells Transfected with pD10-CMV-FGF-18 Plasmid.

Expression of FGF-18 protein was assessed by transient transfection of 293 cells followed by Western analysis, using standard methods. Cell lysates and tissue culture media were harvested at 48 hours post transfection. An anti-peptide FGF-18 rabbit polyclonal antibody, generated against a selected polypeptide from recombinant FGF-18, was used at a dilution of 1:2,500 for one hour at room temperature. The secondary antibody, an anti-rabbit IgG conjugated to peroxidase, was used at a dilution of 1:25,000.

Results of the Western analysis are provided in Figure 8. Briefly, lanes 1-3 represent 1, 2 and 10 ul of tissue culture media from cells transfected with the pD10-CMV-FGF-18 plasmid. Lane 4 is blank. Lanes 5, 6 and 7 contain 2, 10 and 20 ul of lysate from the transfected cells. Lanes 8 and 9 are negative controls; 20 ul of tissue culture media and cell lysate, respectively, from uninfected cells. Lane 10 contains a positive control; an FGF-18-maltose binding protein fusion ((MBP); predicted size = 80 Kd, larger than the FGF-18 protein).

Example H: Packaging of the pD10-CMV-FGF-18 plasmid into rAAV particles.

FGF-18 rAAV virus was generated by the triple transfection method.

EXAMPLE 5

AAV – LacZ INJECTED RETINA

A. Subretinal injection of rAAV

Albino Sprague-Dawley rats were injected at 14 –15 days postnatal (P14- P15). Animals were anesthetized by ketamine/xylazine injection, and a local anesthetic (proparacain HCl) was applied topically to the cornea. An aperture was made through the inferior cornea of the eye with a 28 gauge needle. Subretinal injections of 2-3µl of AAV-CMV-Lac-Z were then made by inserting a blunt 32 gauge needle through the opening and delivering the rAAV suspension into the subretinal space in the posterior retina. The contralateral eye was either uninjected, injected subretinally with PBS, or with control wild-type rAAV containing no reporter gene.

B. Staining Protocol

Bluo-gal staining for b-galactosidase reaction product of lacZ

In all wild type rats tested (3), positive staining was visible in the interior of the whole eyecup upon gross examination (see Figure 9). 100μm thick agarose or 20μm thick cryosections of retinas indicated that most of the b-gal positive staining localized to the photoreceptors. There were a small number of LacZ positive retinal ganglion cells observed.

C. Anti-b-galactosidase immunocytochemistry

Sections from 3 wildtype and 2 transgenic rats were stained with a polyclonal antibody against b-galactosidase. These results were comparable to the bluo-gal results. Primarily demonstrating photoreceptor-specific staining. 2/5 rats showed no positive staining.

D. Results

Subretinal injection of 2 ul of AAV-CMV-lacZ effectively transduced a large number of photoreceptor and retinal pigment epithelial (rpe) cells following a single intraocular innoculation of AAV-CMV-lacZ into the subretinal space (SRS) of the rat eye. The lateral extent of lacZ reporter gene expression was typically 1/3 to 1/2 of the retinal expanse following a single AAV-CMV-LacZ injection. This finding was confirmed by bluo-gal staining of the b-galactosidase reaction product of the lacZ gene as well as by immunocytochemistry using an antibody specific for b-galactosidase. The AAV-CMV-lacZ vector was effective at transducing photoreceptor and RPE cells in both the Normal (wildtype) and affected, degenerating (transgenic) rat retina.

EXAMPLE 6

RETINAL TISSUE ANALYSIS OF RAAV-FGF-2 INFECTED CELLS, VS. CONTROLS

A. Subretinal injection of rAAV

Line 3 albino transgenic rats (P23H-3) on an albino Sprague-Dawley background (produced by Chrysalis DNX Transgenic Sciences, Princeton, NJ) were injected at the ages of P14 or P15. Animals were anesthetized by ketamine/xylazine injection, and a local anesthetic (proparacain HCl) was applied topically to the cornea. An aperture was made through the inferior cornea of the eye with a 28 gauge needle. The subretinal injections of 2 μl were then made by inserting a blunt 32 gauge needle through the opening and delivering the rAAV suspension into the subretinal space in the posterior retina. The intent was to inject into the subretinal space of the posterior

superior hemisphere, but we sometimes found histologically that the injection site was located just inferior to the optic nerve head. The opposite eye was either uninjected, injected subretinally with PBS, with control rAAV containing no neurotrophin or containing proteins not known to possess neurotrophic properties.

B. Histopathology Protocol / Retinal tissue analysis

The rats were euthanized by overdose of carbon dioxide inhalation and immediately perfused intracardially with a mixture of mixed aldehydes (2% formaldehyde and 2.5 % glutaraldehyde). Eyes were removed and embedded in epoxy resin, and 1 μm thick histological sections were made along the vertical meridian. Tissue sections were aligned so that the ROS and Müller cell processes crossing the inner plexiform layer were continuous throughout the plane of section to assure that the sections were not oblique, and the thickness of the ONL and lengths of RIS and ROS were measured as described (LaVail , et al 19XX) . Briefly, 54 measurements of each layer or structure were made at set points around the entire retinal section. These data were either averaged to provide a single value for the retina, or plotted as a distribution of thickness or length across the retina. We also compared the greatest 3 contiguous values for ONL thickness in each retina, to determine if any region of retina (*e.g.*, nearest the injection site) showed proportionally greater rescue; although most of these values were slightly greater than the overall mean of all 54 values, they were no different from control values than the overall mean. Thus, the overall mean was used in the data cited, since it was based on a much larger number of measurements.

C. Results

Two surgical methods of delivery of rAAV-CMV-FGF2 were completed, intravitreal and subretinal injection.

1. Intravitreal injection

RAVV-CMV-FGF-2 was injected into the right eye of nine transgenic S334ter rats after day P15 (the left eye was not injected). S334(4) transgenic animals were used to assess the rescue effect of rAAV-CMV- FGF-2 on degenerating photoreceptor cells when delivered by intravitreal injection. The rats were all sacrificed at age p60 and the embedded in plastic and sectioned to assess morphology and therapeutic effect as assayed by the preservation of thickness of outer nuclear layer. Superior and inferior regions of eyecup are quantitated by measuring the ONL thickness

using a BioQuant morphometric measuring system (BioQuant). Injected eyes were evaluated along with uninjected control eyes.

Control Left superior – 16.52 +/- 2.77 um

Injected Right superior – 19.71 +/- 5.27 um

Control Left inferior – 22.64 +/- 2.11 um

Injected Right inferior – 26.47 +/- 3.55 um

Based upon these results it was evident that there is a rescue effect of AAV-CMV-FGF-2 when delivered intraocularly into the vitreous cavity.

2. Sub-retinal injection of rAAV-CMV-FGF-2

Experiment A. - Location of injection – subretinal , 7 rats-both right and left eyes injected, 3 rats-(left eye = uninjected). Number of rats injected – 10 rats all wild type p15 on day of injection. One rat was sacrificed every week starting at week 2. Expression of FGF-2 was assessed, as well as any signs of inflammation or neovascularization.

Experiment B. - Location of injection – subretinal, 5 rats- right eyes injected w/vector left eyes injected with PBS, 4 rats- right eyes injected w/vector (left eye = uninjected). Number of rats injected – 11 transgenic S334(4) rats – all were p15 on day of injection. The rats were sacrificed at age p60 and the embedded in plastic and sectioned to assess histopathology and number of surviving photoreceptor cells. .

Anatomic indication of therapeutic effect (photoreceptor rescue) was assessed histolotically. Briefly, eyes injected with rAAV-CMV-FGF2 retained significantly more photoreceptors at P60, P75 and P90 than uninjected contralateral control eyes of the same animal. Retinas receiving a subretinal injection of AAV-CMV-FGF2 at P14-15 retained 71% of the normal ONL thickness, compared to about 47% in the uninjected controls (see Figures 12, 13 and 14).

There was little or no rescue in PBS-injected control eyes ($p>0.169$ in all cases). This is consistent with previous reports that needle injury to the retina in young rats (P14-P15) does not rescue photoreceptors or up-regulate bFGF mRNA expression.

3. Interphotoreceptor injection of rAAV-CMV-FGF-2

Two to three microliters of rAAV-CMV-FGF-2 vector was injected into the interphotoreceptor space between the photoreceptors and the adjacent retinal pigment epithelium at P14 or P15. Rats were sacrificed and eyes examined at time points between P60-P90. At these ages in uninjected control eyes of s334ter rats, the ONL thickness, which is an index of photoreceptor cell number, was reduced to about 60% of normal.

Evidence of anatomic rescue was found to be significant to the p=.005 confidence level in retinas transfected by AAV-CMV-fgf-2 when compared to the control AAV vectors or sham injection of PBS by ANOVA (analysis of Variance statistical measures). JMP statistical analysis software (Copyright (c) 1999 SAS Institute Inc. Cary, NC, USA.)

EXAMPLE 7

TRANSGENIC RAT S334TER AS A MODEL FOR PHOTORECEPTOR DEGENERATION

This examples describes the S334ter transgenic rat as a model for photoreceptor degeneration. Briefly, rhodopsin is a seven-transmembrane protein found in photoreceptor outer segments, which acts as a photopigment. The S334ter mutation results in the truncation of the C-terminal 15 amino acid residues of rhodopsin and is similar to rhodopsin mutations found in a subset of patients with retinitis pigmentosa (RP). RP is a heterogeneous group of inherited retinal disorders in which individuals experience varying rates of vision loss due to photoreceptor degeneration. IN many RP patients, photoreceptor cell death progresses to blindness. Transgenic S334ter rats are born with normal number of photoreceptors. The mutant rhodopsin gene begins expression at postnatal day 5 in the rat, and photoreceptor cell death begins at postnatal day 10-15. In transgenic line S334ter-3, approximately 70% of the outer nuclear layer has degenerated by day 60 in the absence of any therapeutic intervention (see Figure 11). The retinal degeneration in this model is consistent from animal to animal and follows a predictable and reproducible rate. This provides an assay for therapeutic effect by morphological examination of the thickness of the photoreceptor nuclear layer and comparison of the treated eye to the untreated (contralateral) eye in the same individual animal.

A. Retinal tissue analysis

The rats were euthanized by overdose of carbon dioxide inhalation and immediately perfused intracardially with a mixture of mixed aldehydes (2% formaldehyde and 2.5 % glutaraldehyde). Eyes were removed and embedded in epoxy

resin, and 1 μm thick histological sections were made along the vertical meridian. Tissue sections were aligned so that the ROS and Müller cell processes crossing the inner plexiform layer were continuous throughout the plane of section to assure that the sections were not oblique, and the thickness of the ONL and lengths of RIS and ROS were measured. These retinal thickness measurements were plotted and establish the baseline retinal degeneration rates for the animal model. The assessment of retinal thickness is as follows: briefly, 54 measurements of each layer or structure were made at set points around the entire retinal section. These data were either averaged to provide a single value for the retina, or plotted as a distribution of thickness or length across the retina. We also compared the greatest 3 contiguous values for ONL thickness in each retina, to determine if any region of retina (*e.g.*, nearest the injection site) showed proportionally greater rescue; although most of these values were slightly greater than the overall mean of all 54 values, they were no different from control values than the overall mean. Thus, the overall mean was used in the data cited, since it was based on a much larger number of measurements.

For AAV-CMV-FGF2 evaluation experiments *in vivo*, we used one line of transgenic rats, TgN(s334ter) line 4 (abbreviated s334ter 4). Expression of the mutated opsin transgene begins at postnatal day P5 in these rats, leading to a gradual death of photoreceptor cells. These rats develop an anatomically normal retina up to P15, with the exception of a slightly increased number of pyknotic photoreceptor nuclei in the outer nuclear layer (ONL) than in non-transgenic control rats. In this animal model , the rate of photoreceptor cell death is approximately linear until P60, resulting in loss of 40-60% of the photoreceptors. After P60, the rate of cell loss decreases, until by one year the retinas have less than a single row of photoreceptor nuclei remaining.

EXAMPLE 8

ANTIBODY STAINING OF RAAV-FGF-2 INFECTED CELLS

A. Injection Protocol

Albino Sprague-Dawley rats were injected with rAAV-FGF-2 at the ages of P14 or P15 essentially as follows. Briefly, wild-type animals were anesthetized by ketamine/xylazine injection, and a local anesthetic (proparacain HCl) was applied topically to the cornea. An aperture was made through the inferior cornea of the eye with a 28 gauge needle. The subretinal injections of 2-3 ul of AAV-CMV-FGF-2 were then made by inserting a blunt 32 gauge needle through the opening and delivering the rAAV suspension into the subretinal space in the posterior retina. The contralateral eye

was either uninjected, injected subretinally with PBS, wild-type rAAV, or with AAV-CMV-lacZ.

B. Staining Protocol

Fixed eyecups were embedded in OCT and cryosectioned in 20um thick sections. Sections from 10 wt rats were stained with antibody to FGF-2. (primary- anti-FGF-2 1:500 (commercial antibody purchased from R&D systems) (secondary-anti-goat Cy3 conjugate (Sigma, St. Louis. MO).

C. Results

Immunohistochemistry was used to look for expression of FGF-2 in the eye. Two rats were examined every week starting at 3 weeks postinjection. Retinas were examined for expression of FGF-2 and also examined histopathologically for signs of inflammation or neovascularization.

Results are shown in Figure 15. Briefly, expression of FGF-2 was found in retinal photoreceptor cells at 35 days following innoculation with 2-3 ul of rAAV-CMV-FGF-2. Less significant expression was noted in retinal bipolar interneurons and retinal ganglion cells (RGCs) following injection into the subretinal space (SRS). No significant staining above background was observed in sections injected with PBS or rAVV-CMV-lacZ vectors.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

CLAIMS

We claim:

1. A method of treating or preventing retinal diseases of the eye, comprising, administering intraocularly a gene delivery vector which directs the expression of a neurotrophic factor, such that said retinal disease of the eye is treated or prevented.

2. The method according to claim 1 wherein said neurotrophic factor is NGF, BDNF, CNTF, NT-3, or, NT-4.

3. The method according to claim 1 wherein said neurotrophic factor is a FGF.

4. The method according to claim 3 wherein said FGF is FGF-2, FGF-5, or, FGF-18.

5. The method according to claim 1 wherein said retinal disease of the eye is macular degeneration.

6. The method according to claim 1 wherein said retinal disease of the eye is diabetic retinopathy.

7. The method according to claim 1 wherein said retinal disease of the eye is an inherited retinal degeneration.

8. The method according to claim 7 wherein said inherited retinal degeneration is retinitis pigmentosa.

9. The method according to claim 1 wherein said retinal disease of the eye is glaucoma.

10. The method according to claim 1 wherein said retinal disease of the eye is a surgery-induced retinopathy.

11. The method according to claim 1 wherein said retinal disease of the eye is retinal detachment.

12. The method according to claim 1 wherein said retinal disease of the eye is a photic retinopathy.

13. The method according to claim 1 wherein said retinal disease of the eye is a toxic retinopathy.

14. The method according to claim 1 wherein said retinal disease of the eye is a trauma-induced retinopathy.

15. The method according to claim 1 wherein said gene delivery vector is a retrovirus selected from the group consisting of HIV and FIV.

16. The method according to claim 1 wherein said gene delivery vector is a recombinant adeno-associated viral vector.

17. A gene delivery vector which directs the expression of a neurotrophic factor.

18. The gene delivery vector according to claim 17 wherein said neurotrophic factor is NGF, BDNF, CNTF, NT-3, or, NT-4.

19. The gene delivery vector according to claim 17 wherein said neurotrophic factor is a FGF.

20. The gene delivery vector according to claim 17 wherein said FGF is FGF-2, FGF-5, or, FGF-18.

21. The gene delivery vector according to claim 17 wherein said vector is generated from a retrovirus.

22. The gene delivery vector according to claim 21 wherein said retrovirus is HIV or FIV.

23. The gene delivery vector according to claim 17 wherein said vector is generated from a recombinant adeno-associated virus.

USE OF RECOMBINANT GENE DELIVERY VECTORS FOR TREATING OR PREVENTING DISEASES OF THE EYE

ABSTRACT OF THE DISCLOSURE

Gene delivery vectors, such as, for example, recombinant adeno-associated viral vectors, and methods of using such vectors are provided for use in treating or preventing retinal diseases of the eye.

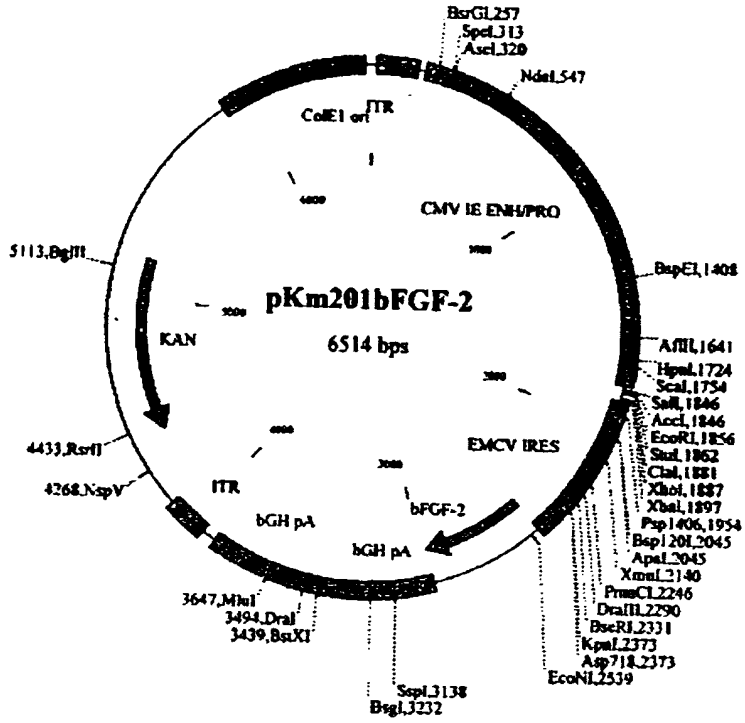

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SalI,1846
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StuI,1862
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KpnI,2373
Asp718,2373
EcoNI,2539
SspI,3138
BsgI,3232
3647,MluI
3494,DraI
3439,BsaXI
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bFGF-2
EMCV IRES
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4268,NspV
KAN
5113,BglII

Fig. 1

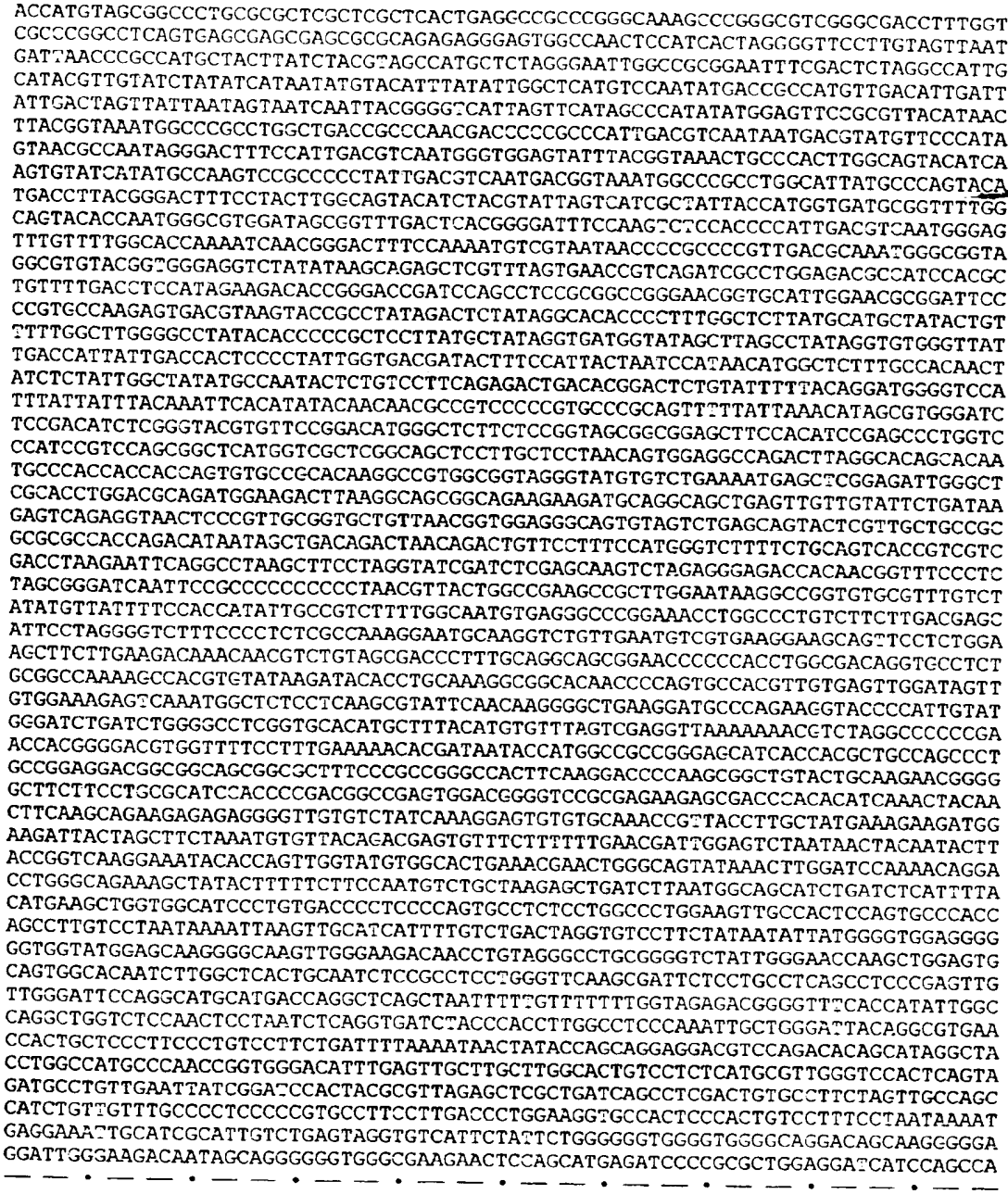


Fig. 2A

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Fig. 2B

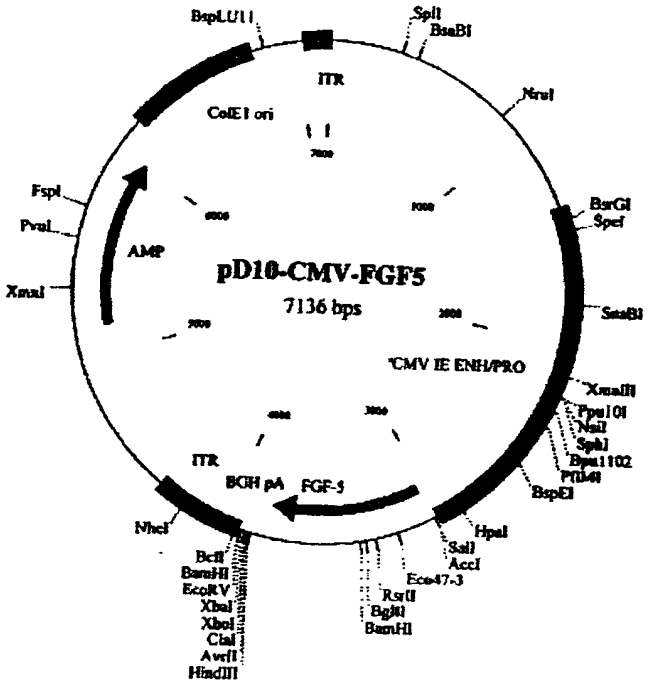

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BsrGI
SpeI
SnaBI
CMV IE ENH/PRO
XmaIII
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SalI
AccI
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ClaI
AvrII
HindIII

Fig. 3



Fig. 4

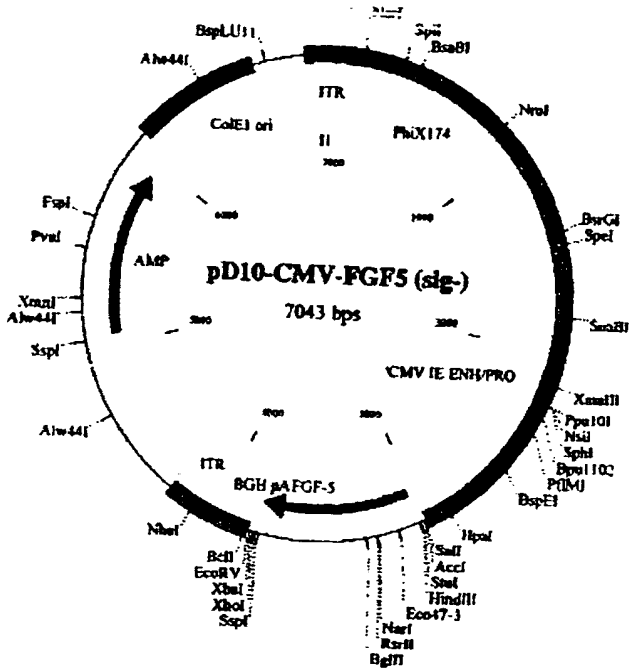

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Fig. 5

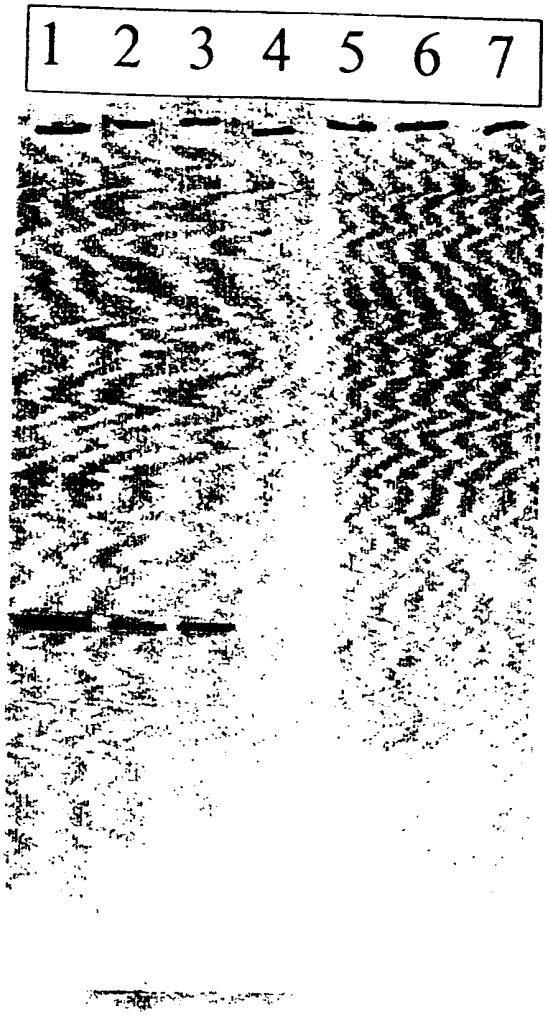


Fig. 6

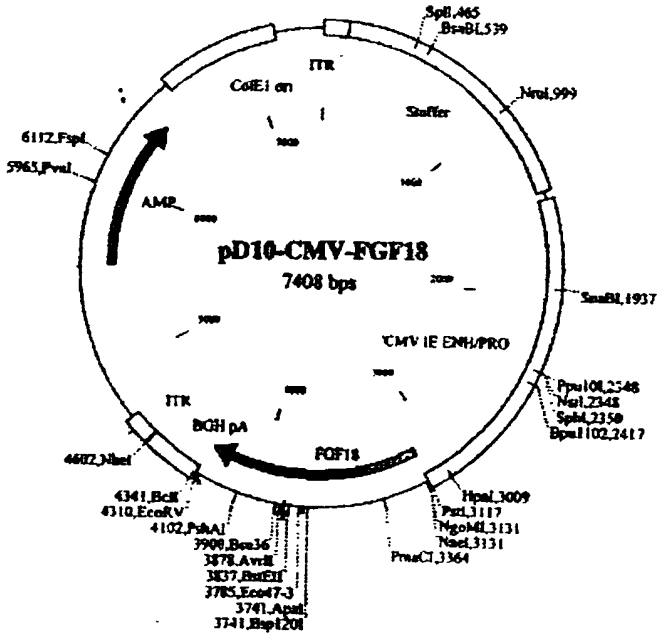

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Fig. 7

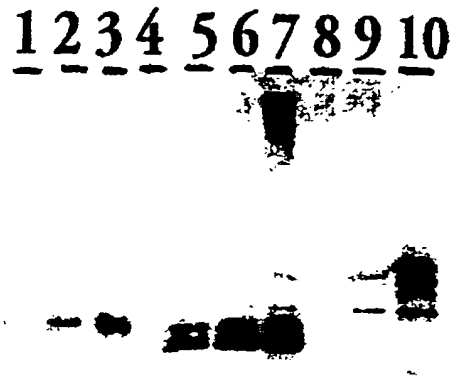


Fig. 8



Fig. 9

SUBSTITUTE SHEET

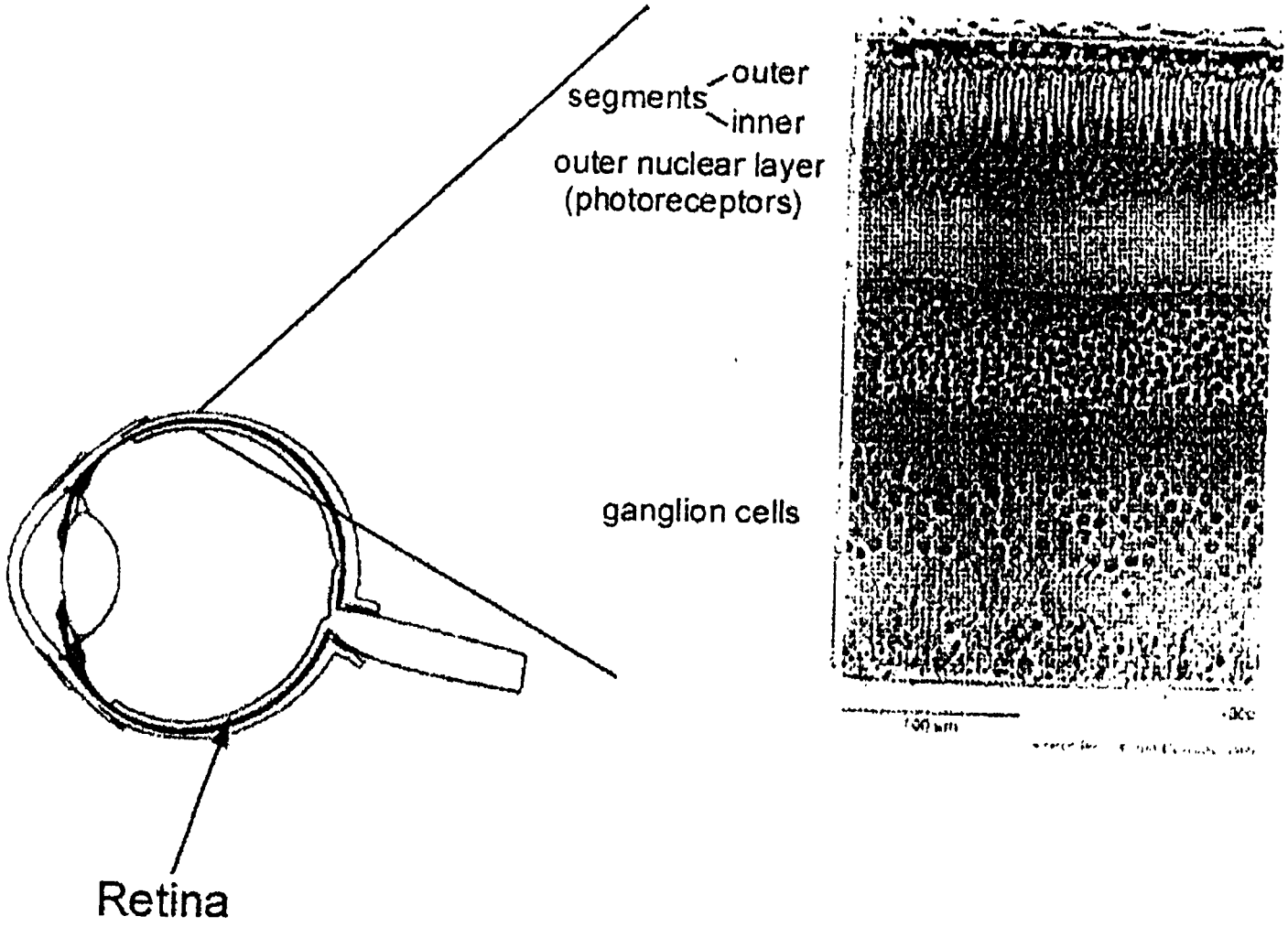


Fig. 10

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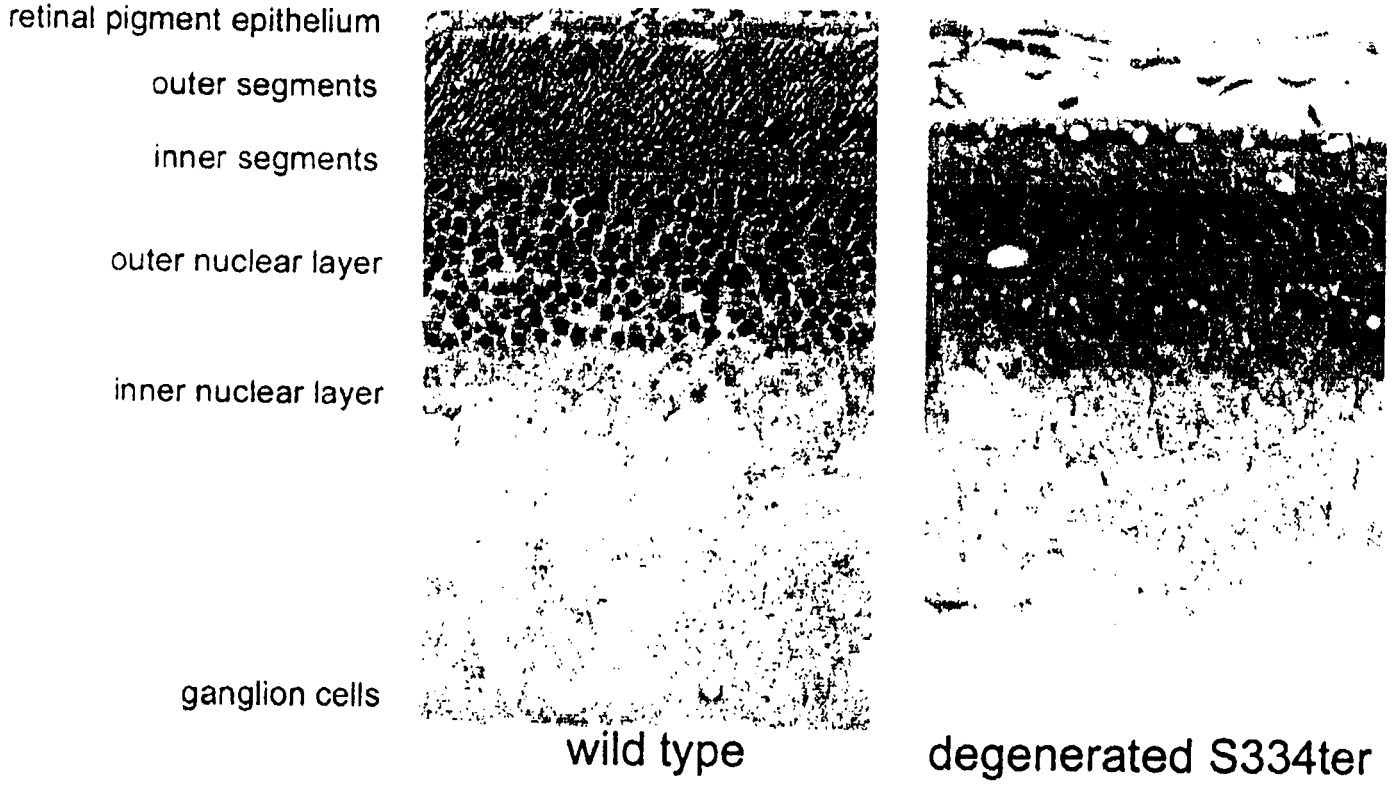


Fig. 11

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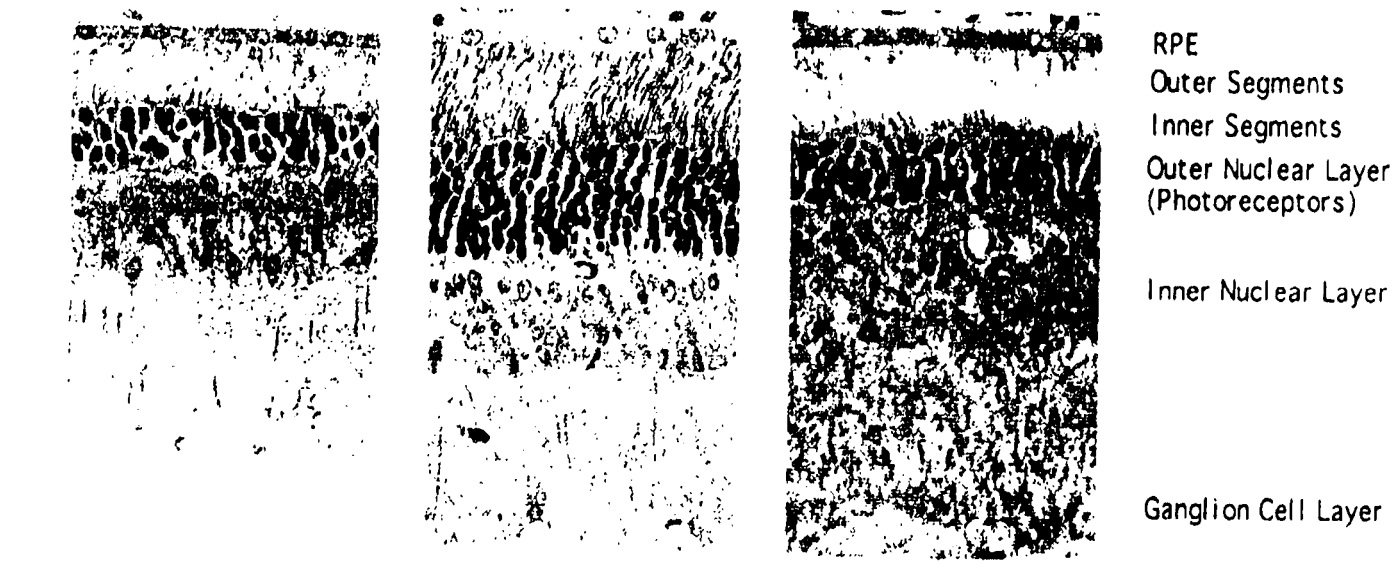


Fig. 12

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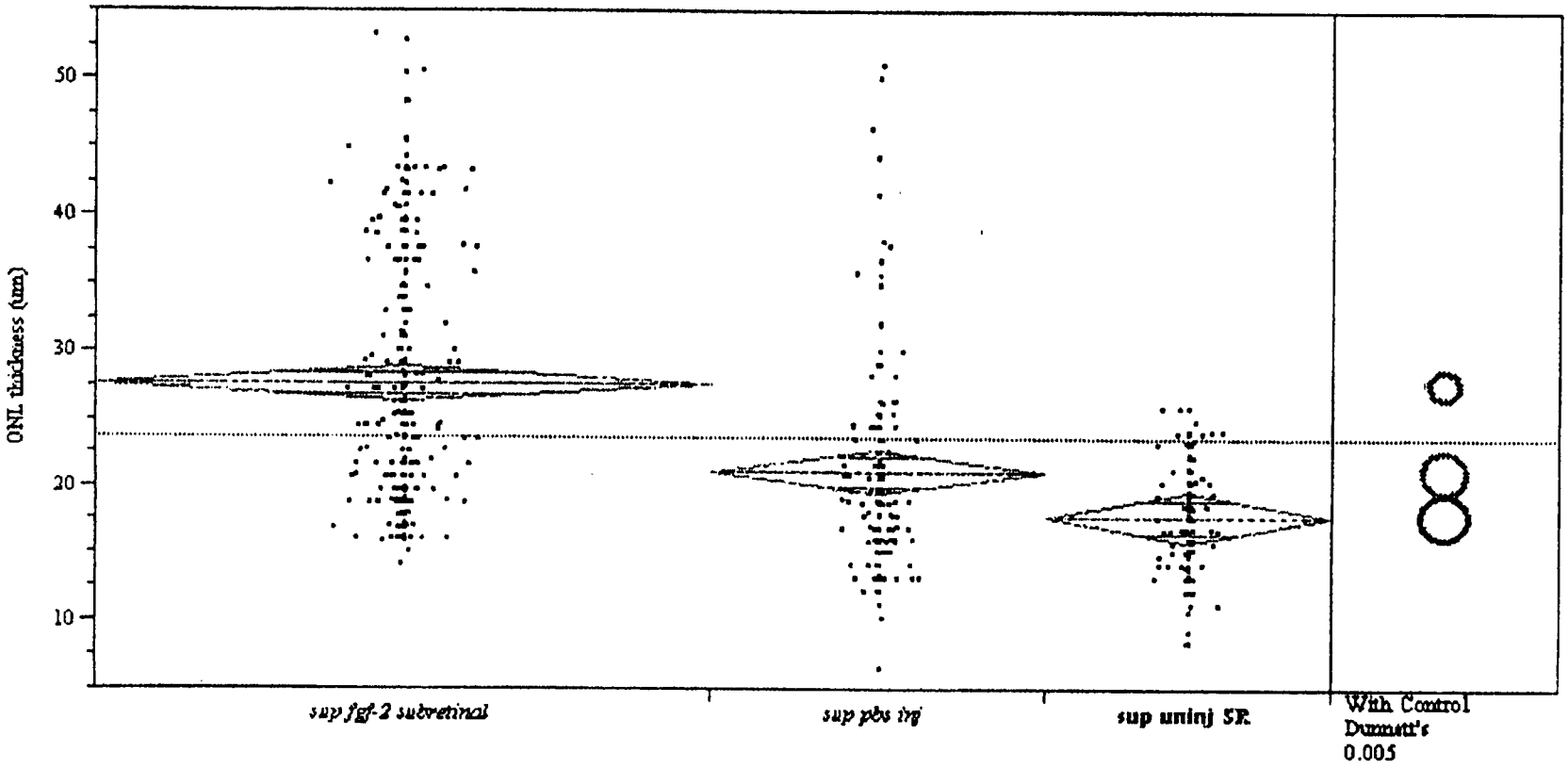


Fig. 13

Boston Otherss

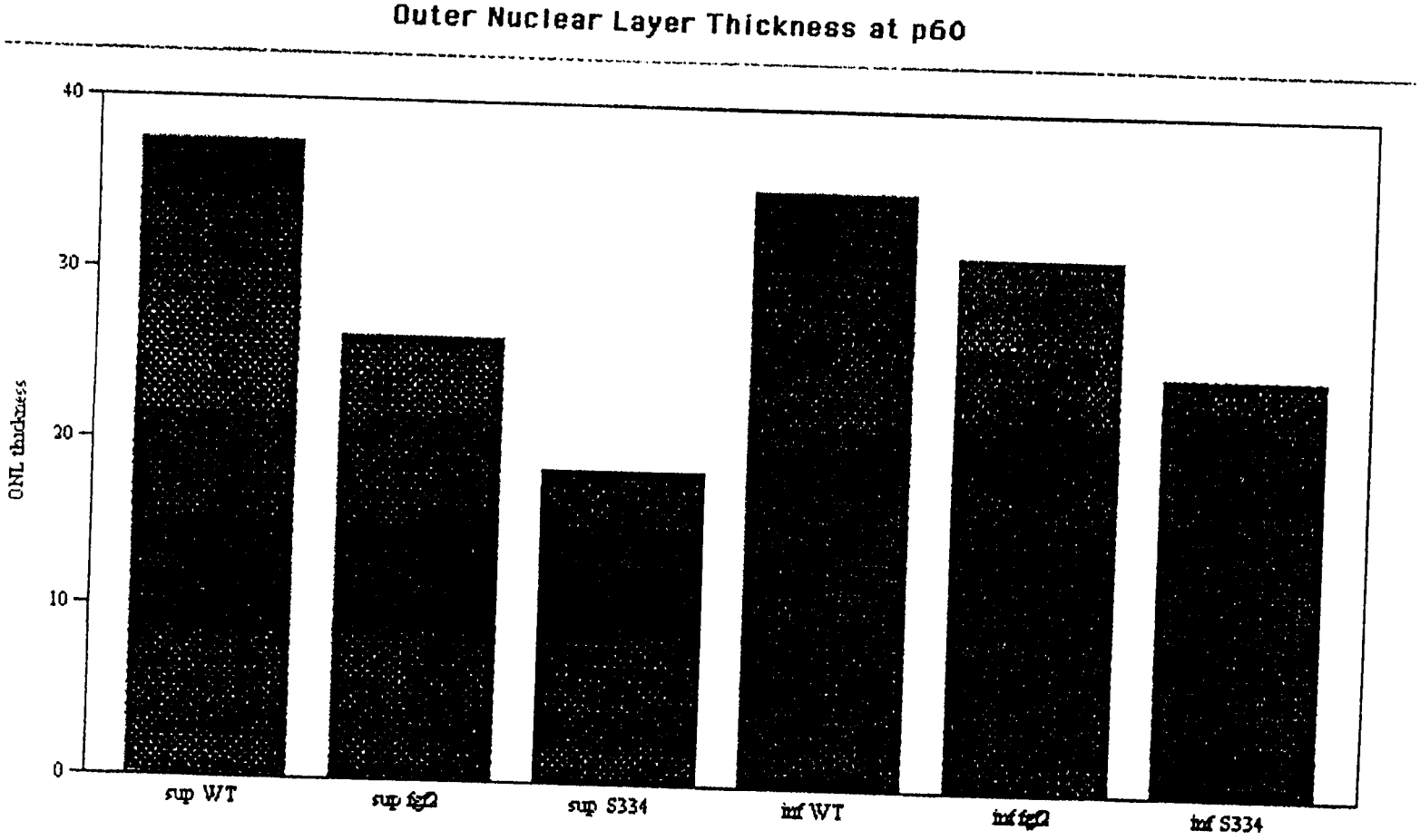


Fig. 14

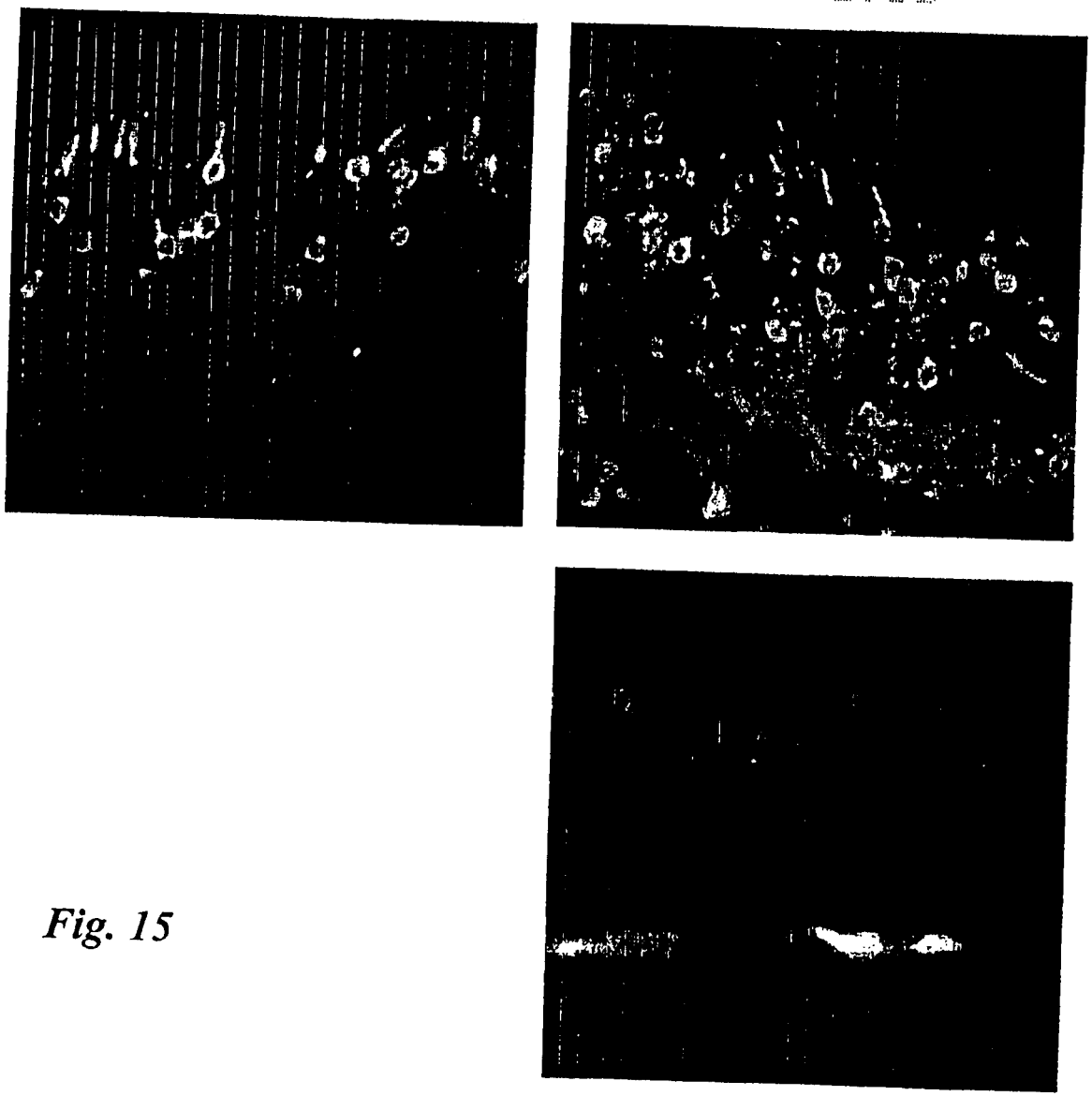


Fig. 15